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Published in:
Parasite Genomics Protocols

Publication date:
2004

Citation for published version (APA):
Hoffmann, K. F., & Fitzpatrick, J. M. (2004). Gene Expression Studies Using Self-Fabricated Parasite cDNA Microarrays. In S. E. Melville (Ed.), *Parasite Genomics Protocols* (270 ed., pp. 219-236). Springer Nature. http://hdl.handle.net/2160/5783

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Gene Expression Studies Using Self-Fabricated Parasite cDNA Microarrays

Karl F. Hoffmann and Jennifer M. Fitzpatrick

Summary

DNA microarray platforms represent a functional genomics technology that uses structured information obtained from genomic sequencing efforts as a means to study transcriptional processes in a systematic and high-throughput manner. Specifically in this chapter, we outline the ordered processes involved in large-scale parasite gene expression studies including complementary (cDNA) microarray fabrication, total RNA isolation, cDNA labeling using fluorochromes, and DNA:DNA hybridization. Methods described herein were adapted for the study of schistosome sexual maturation and developmental biology but could be easily modified for the study of any additional parasitological system.

Key Words: cDNA; DNA microarray; functional genomics; gene expression; hybridization; parasite; RNA.

1. Introduction

Continued funding of parasite genome sequencing projects has led to the successful cataloging of several hundred thousand expressed sequence tags (ESTs) ***(1)*** from many different organisms. ESTs (short stretches of DNA sequence derived from cloned complementary DNAs [cDNAs]) have proved to be an invaluable starting resource for gene discovery projects ***(2–7)*** in many laboratories across the world and have contributed to our understanding of diverse biological processes. However, despite the obvious advantages of scanning large databases containing individual EST sequences for specific gene identification purposes, there is limited information to be obtained with this "piecemeal" approach for subsequent interpretation of gene expression studies in the modern genomic age. For example, it would require years of gene sequence analysis and several rather complicated experimental gene expression techniques (yeast 2 hybrid protocols, GST-capture assays, etc.) to dissect the related components

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From: *Methods in Molecular Biology, Vol. 270: Parasite Genomics Protocols*
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of a signaling cascade in *Brugia malayi* from the starting point of a putative microfilarial chemokine receptor identified via the filarial EST databases (http://circuit.neb.com/fgn/filgen1.html). Additionally, if one were interested in the gene transcription pathways associated with sexual dimorphism in schistosome biology, one would find it difficult to ascertain this information via traditional experimental techniques (subtractive cDNA libraries, differential display, etc.) or querying the existing schistosome EST databases (http://www.nhm.ac.uk/hosted_sites/schisto/). Clearly, a more global approach to the study of gene expression is needed to relate ESTs to other gene products in complicated biological systems. With the advent and refinement of cDNA microarray technology, expression analysis and functional relationship studies can now be easily initiated, which, as a result, has led to a revolution in the way gene discovery projects are performed in the 21st century.

cDNA microarray platforms useful for gene expression studies are a vital component of functional genomics, as protein microarrays will be a component of functional proteomics. Functional genomics is a catch-all term used to describe a research area in which EST or genomic sequence information is used to decipher the *functional* meaning of *genomes*. A cDNA microarray containing different EST clones allows for a high-parallel, simultaneous, high-throughput screening strategy from very small amounts of starting material. Essentially, cDNAs identified through parasite EST sequencing efforts can be arrayed in spatially defined grid patterns. High densities of cDNA "spots" can be achieved on chemically modified glass microscope slides (although membranes can be used, the advantages of glass over membrane are discussed elsewhere ***[8]***) through the use of a programmable robot. These cDNA microarrays are then used as a probe (by convention—probes are the spotted DNA on the array and target is the cDNA generated from each RNA pool) to assess the relative abundance of gene transcripts between two separate RNA target pools using Southern hybridization procedures ***(9)***. In addition to single bimodal types of experimental assays, cDNA microarrays can also be used to study the longitudinal changes in parasite gene transcription in response to a physiological stress, drug, or immunological pressure.

This chapter describes the procedures (*see* **Fig. 1** for a flow diagram representation) essential for the successful design of cDNA microarray experiments

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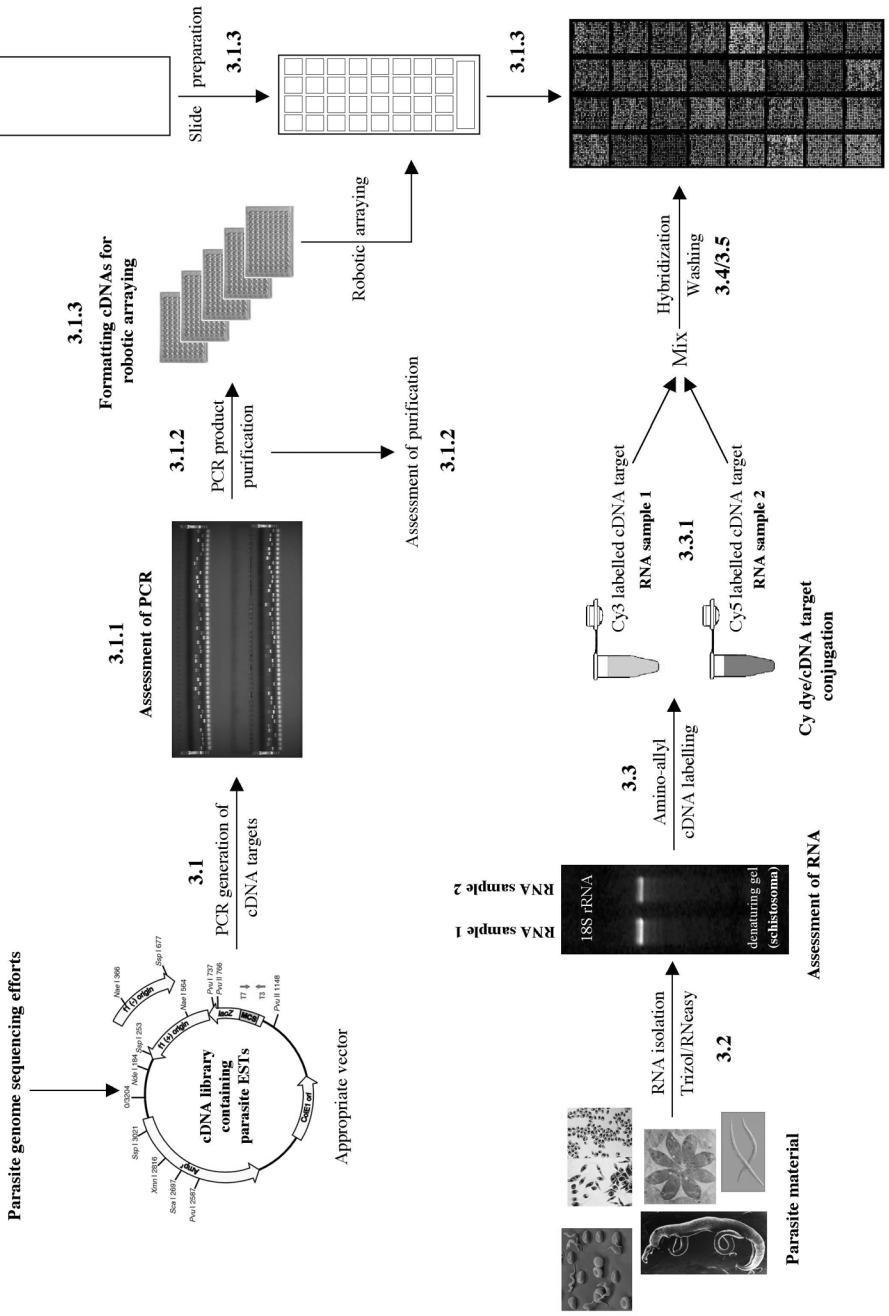
Fig. 1. (*Opposite page*) Flow diagram representation of processes and procedures involved in cDNA microarray gene-expression studies. Each **bold** number (3.1–3.5) indicates a subheading in **Section 3.** in the Methods portion that contains detailed instructions for successful completion of the pictured or described procedure. Vector map of pBluescript II SK+ was obtained from Stratagene and individual parasite images from the World Wide Web.



for use in parasitological-related gene expression studies. Techniques and protocols that have been used successfully in studies on schistosome-host immunobiology are included, although modifications to these protocols used by other investigators have been mentioned where applicable. The following techniques have been modified and adapted from protocols published within the cDNA microarray community and are cited as such. Intentionally excluded are the fine details of data mining and bioinformatics, as this in itself would require a separate discussion and is an ever-evolving mathematical and statistical discipline.

2. Materials

2.1. cDNA Probe Amplification, Purification, and Printing

1. Oligonucleotide polymerase chain reaction (PCR) primers to amplify cDNA probes (from any commercial source).
2. Standard PCR reagents and thermal cycler (capable of handling 96-well PCR plates).
3. Standard 96-well PCR plates.
4. Self-adhering PE foil (Macherey-Nagel 740676).
5. NucleoFast 96-well filter plates (Macherey-Nagel 7435000.4).
6. Vacuum manifold (e.g., Millipore/MultiScreen, Qiagen/QIAvac 96, Promega/VacMan 96, BioRad/Aurum).
7. Centrifuge alignment frame (Millipore MACF09604).
8. 96-well V-bottom plates (e.g., Nunc 442587).
9. 384-well V-bottom plates (e.g., Genetix X7022).
10. Bar-coded γ-amino propyl silane-coated slides (Corning 40003).
11. 4× Spotting buffer (600 m*M* sodium phosphate; 0.04% sodium dodecyl sulfate [SDS]): 48.9 mL 600 m*M* Na_2HPO4, 1.1 mL 600 m*M* NaH_2PO4, 200 µL 10% SDS (*see* **Note 1** for alternative spotting buffers).
12. Printing tool (and pins).
13. Robotic printer: MicroGrid II, BioRobotics (biorobotics.co.uk); GeneTac G3, Genomics Solutions (also incorporating GeneMachines and Cartesian Technologies); Genetix "Q" Array (genetix.co.uk); LabMan HDMS (labman.co.uk).
14. UV stratalinker (Stratagene).
15. 80°C oven.

2.2. RNA Isolation (Everything Must Be RNAse-Free)

1. Trizol (Invitrogen 15596-018).
2. Rotor stator homogenizer (Tekmar TISSUMIZER or equivalent).
3. Chloroform.
4. Ethanol (EtOH).
5. RNeasy column chromatography kits (Qiagen: midi kit 75142, maxi kit 75162).
6. 3 *M* sodium acetate, pH 5.2.
7. 1 *M* Tris-HCl, pH 7.4.

8. RNase-free H_2O.
9. Filter barrier pipet tips.

2.3. cDNA Labeling, Target Cleanup, Hybridization, and Slide Washing

1. Superscript II reverse transcriptase, SSRT II (10,000 U/mL) (Invitrogen 18064-014).
2. Oligo-dTV primer (1 μ*M* scale synthesis) 5'-TTT TTT TTT TTT TTT TTT TTV-3' (V designates any other deoxynucleotide except dT).
3. Microcon-YM30 concentrators (30,000 MW cutoff) (Amicon 42410).
4. Deoxynucleotides (100 m*M* each) (Pharmacia: dATP 272-050, dCTP 272-060, dGTP 272-070, and dTTP 272-080).
5. 5-(3-Aminoallyl)-2'-dUTP (1 mg) (Sigma A0410).
6. Cy3 mono-reactive dye pack (5 mg) (Amersham PA23001). Resuspend individual Cy dye tubes (5 tubes/pack) in 10 μL dimethyl sulfoxide (DMSO). Aliquot 1.25 μL to eight separate tubes. Evaporate dyes in Speed-Vac in dark. Store samples under vacuum in a dessicator at 4°C in dark.
7. Cy5 monoreactive dye pack (5 mg) (Amersham PA25001). Prepare as above prior to use.
8. 0.5 *M* EDTA, pH 8.0.
9. 1 *M* NaOH.
10. 20× SSC.
11. DMSO.
12. 10% SDS.
13. 50× amino-allyl (aa)- dUTP/dNTP mix (2:3 ratio of aa-dUTP/dTTP): 10 μL dATP (100 m*M* stock), 10 μL dCTP (100 m*M* stock),10 μL dGTP (100 m*M* stock), 6 μL dTTP (100 m*M* stock), 4 μL aa-dUTP (100 m*M* stock).
14. Master mix for cDNA labeling (for 50 rxns): 300 μL SSRT II 1st strand cDNA buffer (supplied with SSRT II enzyme), 150 μL 0.1 *M* dithiorthreitol (DTT) (supplied with SSRT II enzyme), 150 μL RNAse-free H_2O, 30 μL (50×) amino-allyl (aa)-dUTP/dNTP mix (2:3 ratio of aa-dUTP/dTTP).
15. 100 m*M* sodium bicarbonate ($NaHCO_3$).
16. 100 m*M* sodium carbonate anhydrous (Na_2CO_3). Mix 100 m*M* $NaHCO_3$ and 100 m*M* Na_2CO_3 together until final pH 8.5–9.0 is achieved.
17. 4 *M* hydroxylamine (Sigma H9876).
18. Qiaquick PCR purification kit (Qiagen 28104).
19. $pd(A)_{40–60}$ (Amersham 27-7988-01) (reconstituted at 8 mg/mL).
20. Yeast transfer RNA (tRNA) (Sigma R8759) (reconstituted at 4 mg/mL).
21. Bovine serum albumin (BSA) (Fraction V: Sigma).
22. 50× Denhardt's reagent.
23. Deionized formamide.
24. Sodium pyrophosphate.
25. 1 *M* Tris-HCl (pH 7.4).
26. Isopropanol.
27. Hybridization chambers (ArrayIt™ AHC-1 or equivalent).

AU: Pls spell out SSC at first use with abbreviation in parens.

28. Lifter-slips (Erie Scientific: size depends on cDNA array dimensions)/coverslips (size depends on cDNA array dimensions).
29. Coplin jars.
30. Slide swing handle rack (RA Lamb E102).
31. Cot-1 DNA; mouse and human (Invitrogen).

2.4. Scanning/Software

1. Scanner: e.g., GenePix 4000B, Axon (http://www.axon.com); ScanArray 3000, Packard BioChip Technologies/GSI Lumonics. (http://www.dssimage.com/microarraySystems.html); Avalanche, Molecular Dynamics (Amersham Biosciences) (http://www.mdyn.com); DNAscope, GeneFocus (http://www.genefocus.com).
2. Spot-finding software: e.g., GenePix Pro, Axon (http://www.axon.com); Microarray Suite, Scanalytics (http://www.scanalytics.com).
3. Data-mining software: e.g., Spotfire DecisionSite for Functional Genomics, Spotfire, (http://www.spotfire.com); Genespring, Silicon Genetics (http://www.silicongenetics.com).

3. Methods

3.1. cDNA Probe Amplification, Purification, and Printing

3.1.1. Amplification

cDNA probes to be robotically printed onto chemically modified glass slides are generated according to standard PCR protocols (*see* **Note 2**). Briefly, a 100-µL reaction amplified for 35 cycles generally is sufficient to generate adequate probe material to be spotted onto several hundred glass slides. For ease in processing multiple samples, 96-well PCR plates are recommended for the amplification. The source of template used for each unique parasite cDNA typically is a phage suspension or a lysed bacterial colony (obtained from cDNA libraries or cloned individually). Usually, these clones have been sequence verified. There is no need to isolate purified DNA (containing parasite-specific cDNA) before proceeding with the PCR reactions unless problems arise with the amplification (*see* **Note 3**). PCR primers used in the amplification are either complementary to cDNA vector sequences, flanking the parasite-specific inserts in the starting library (e.g., T7/T3, M13R/M13F), or are gene-specific.

Analyze a 3-µL sample from each PCR reaction on a 1% agarose gel to verify size and number of products (*see* **Fig. 1**). The remaining PCR reactions may be stored at –80°C until purification can be performed.

3.1.2. Purification

Before the parasite-specific cDNA probes are suitable for printing, they must be purified away from all contaminating salts, dNTPs, and primers. An

easy and efficient way to achieve high-throughput purification of multiple samples is via the use of NucleoFast® 96-well filter plates (Macherey-Nagel).

1. Transfer each 100-µL PCR reaction directly onto the membrane of the NucleoFast 96-well filter-plate placed on top of an appropriate vacuum manifold (with waste collection plate) (*see* **Note 4**).
2. To remove contaminants by ultrafiltration, apply a vacuum of –400 to –600 mbar[2] (reduction of atmospheric pressure), typically for 10–15 min (NO modifications to this protocol are necessary if mineral oil was used during the PCR reactions, although the actual filtration time may be longer).
3. Dispense 100 µL nuclease-free double-distilled H_2O (ddH_2O) to each well of the 96-well filter plate while applying the vacuum until the water has passed through the membrane. Repeat this wash two additional times and completely ventilate the manifold for 60–90 s.
4. To recover the DNA, add 100 µL nuclease-free ddH_2O (ensure no vacuum is applied) and transfer filter plate to a suitable microplate shaker with moderate shaking for 10 min (*see* **Note 5**). Gently remove the water from the filter plate wells with a multichannel pipet and dispense into the supplied elution 96-well plates. This will deliver the PCR product at the original concentration. A precipitation step may additionally be included at this point if the final concentration of DNA is required to be higher (*see* **Note 5**). It is advisable to check purification procedure by analyzing PCR products (approx 1 µL) on a 1% agarose gel. Purified products can be stored at –80°C in 96-well plates (source plate) that have been sealed with self-adhering PE foil and the lid safely taped to prevent evaporation.

3.1.3. Printing

1. A small aliquot (15 µL) of the total purified PCR product (from source plate) should be transferred to a new V-bottomed 96- or 384-well plate (print plate).
2. Add 5 µL (4×) spotting buffer to each well and mix thoroughly.
3. Print PCR products onto γ-amino propyl silane-coated slides according to robotic arrayer instructions (*see* **Note 6**). Be sure to include appropriate controls (which can be added to empty wells of print plate) including, but not all-inclusive: genomic DNA (positive control), yeast tRNA (negative control), buffer only (negative control), plasmid and phage DNA from which PCR products were amplified (negative controls).
4. After printing, slides should be allowed to dry at room temperature, baked for 2 h at 80°C and UV cross-linked (600-mJ dose) to facilitate maximal fixation of PCR products. At this stage, if the slides used for printing are not pre-bar coded, then generally it is useful to etch pertinent information into each glass slide with a diamond scribe (while salt residue still defines the border of the array). This information should include slide number, print series, and borders of the printed array. Marking each slide in this manner also serves to distinguish the side of glass that contains DNA.

5. After marking glass slides, denature immobilized PCR products by submerging in a boiling dH_2O bath (turn heating unit off immediately after slides have been submerged) for 2 min.
6. After denaturing DNA, transfer slides to a 95% EtOH bath and fix for 1 min. Spin-dry slides for 5 min at 1000*g* in a benchtop centrifuge. Slides can now be stored in a dust-free microscope slide box at room temperature (*see* **Note 7**) and are ready for hybridization.

3.2. Total RNA Isolation and Purification

As in all applications that involve the isolation of RNA, care must be taken to avoid introduction of ubiquitous RNAses. Either purchase RNAse/DNAse free reagents (preferred) or treat all solutions (except those containing Tris) thoroughly with diethyl-pyrocarbonate (DEPC) prior to use. Change gloves frequently, and use filter tips and disposable plastics when isolating RNA from parasite material. Generally, it is best to work rapidly during the isolation of RNA (the RNA isolation procedure should be performed in 1 d). The method outlined below produces total RNA free from contaminating carbohydrates and protein and involves the use of both TRIZOL Reagent (Invitrogen) and Qiagen RNeasy affinity columns (Qiagen). RNA isolated in other fashions (although suitable for Northern blotting, RNAse protection, or reverse transcriptase [RT]-PCR assays) generally is not suitable for cDNA microarray applications and can often lead to high background because of biological contaminants. Extremely low amounts of genomic DNA may be isolated during the procedure described here. If this presents a problem (there are reports that Superscript reverse transcriptase II can prime off genomic DNA, leading to false positives; *see* Genisphere's Web site http://www.genisphere.com). Qiagen provides a protocol for genomic DNA digestion on a column, or alternatively, one can follow any protocol that uses the enzyme RNAse-free DNAse.

3.2.1. TRIZOL Extraction

According to the manufacturer's instructions, add a sufficient volume of TRIZOL to parasite material (1 mL of TRIZOL per 10^7 cells or 1 mL TRIZOL per 100 mg tissue—*see* **Note 8**). If starting from parasite-infected cell cultures (e.g., *Toxoplasma gondii*-infected fibroblasts or *Plasmodium* sp.-infected red blood cells), then refer to commonly used techniques to enrich for parasite material (if desired). Keep in mind that there is a trade-off between obtaining increased quantities of purified parasite material (enriching away from host material) vs the effect this enrichment has on parasite transcriptional processes. Each researcher will have his or her own protocols for obtaining parasite material (fresh material is best, especially if old parasite material was stored improp-

erly), as well as his or her own favorite method of disrupting/homogenizing samples. For multicellular parasites (helminths, nematodes, or other large metazoans), a homogenizer works quite well for this important application.

1. Using a cleaned homogenizer (clean with 50 mL ddH_2O, 50 mL 100% EtOH, and 7 mL TRIZOL between each parasite sample), thoroughly disrupt parasite material (10–15 s). Make sure the tip of the homogenizer stays below the surface of TRIZOL to avoid foaming of solution. After complete homogenization/disruption of sample material, let the sample sit at room temperature for 5 min.
2. Add 0.2 mL chloroform per 1 mL TRIZOL and vigorously shake samples for at least 1 min (*see* **Note 9**). Let samples incubate at room temperature for 2 min to ensure the complete dissociation of nucleoprotein complexes.
3. Spin samples to separate phases (4°C at 12,000*g* for 15 min) (*see* **Note 10**).
4. Carefully transfer aqueous supernatant (containing the RNA) to a new RNAse-free tube without disturbing the interface (containing protein and other cellular debris) and proceed with Qiagen RNeasy affinity purification.

3.2.2. Qiagen Affinity Purification

The second phase of the total RNA purification procedure is to use a Qiagen affinity column to reversibly bind the nucleic acid from the aqueous solution obtained above (see Qiagen RNeasy handbook for detailed principles and procedures of product).

1. Add an equal volume of 70% EtOH to the aqueous supernatant obtained from the TRIZOL extraction (dripwise while vortexing gently to avoid local RNA precipitation) (*see* **Note 11**).
2. Transfer this solution to a Qiagen affinity column. If the volume of solution is greater than the capacity of the Qiagen column, add the sample in successive aliquots. Spin for 5 min at room temperature (3000–5000*g*) to facilitate RNA binding.
3. Vortex the collected flow-through (FT) and reapply to Qiagen column. Spin again for 5 min at room temperature (3000–5000*g*). This step ensures that all RNA in the sample is bound to the column.
4. Discard the FT and stepwise wash the column as indicated in the Qiagen handbook. Volumes of washing buffers will depend on Qiagen column used (midi or maxi column).
5. After the last wash in buffer RPE (supplied in the Qiagen kit) and ensuring that the Qiagen column is completely dry (additional 5 min spin after removal of last FT), carefully transfer the column to a new conical tube (supplied in the Qiagen kit). Elute RNA from the column with three successive equal volumes of RNAse-free ddH_2O (also supplied in the kit) (*see* **Note 12**). All three samples containing the eluted RNA can be collected in the same collection tube.
6. Add 1/10 vol of 3 *M* sodium acetate (pH 5.2–5.3) and 2.5 vol of 100% EtOH to the RNA sample. Vortex this mixture and incubate on ice for at least 10 min (alternatively, place at –20°C overnight to facilitate precipitation of RNA). Spin pre-

cipitated RNA at 12,000*g* for 15 min (4°C). You may have to transfer sample to several 1.5-mL microcentrifuge tubes.

7. Wash pellets twice with 75% EtOH by vigorously vortexing sample during each wash. Dry pellets completely in Speed-Vac (*see* **Note 13**).
8. Dissolve RNA pellet(s) from each sample in RNAse-free ddH_2O by adding directly to the top of the dried sample and placing at 65°C for 10 min (it may be necessary to incubate longer at 65°C). Do not pipet the sample up and down (RNA may stick to the inside of the pipet tip). After the sample has completely dissolved, combine all aliquots of each sample (if necessary) and transfer to a new microcentrifuge tube. Spin out any insoluble debris (3 min at max rpm in microcentrifuge) and quantitate each RNA sample by spectrophotometry (*see* **Note 14**). Aliquot RNA (10-µg aliquots) into RNAse-free microcentrifuge tubes and store samples at –80°C. It is advisable to check the integrity of the RNA sample by electrophoresis through a denaturing agarose gel (*see* **Fig. 1**).

3.3. cDNA Target Labeling With Amino-Allyl dUTP

Although there are alternative cDNA-labeling methods used in cDNA microarray applications (see http://www.nhgri.nih.gov/DIR/Microarray/main.html and http://www.genisphere.com for information on two additional techniques), the protocol listed below describes a procedure that uses amino-allyl dUTP and the fluorescent dyes Cy3 and Cy5. This cDNA-labeling protocol has been slightly modified from one that is available at http://www.microarrays.org (the original protocol was developed at Rosetta Inpharmatics, Kirkland, WA), with the user supplying all the reagents (alternatively, kits that utilize a similar procedure can be purchased from commercial sources—e.g., Fairplay system from Stratagene). As little as 8 µg of total RNA can be used to generate fluorescently modified cDNA molecules of sufficient quantity and intensity to be used in cDNA microarray hybridizations. The protocol detailed below uses 10 µg of total RNA as the starting input material for each separate cDNA reaction (Cy3 and Cy5). Empirical testing of input RNA is recommended for optimal results (generally, more input RNA leads to generation of cDNA with higher signal intensity).

3.3.1. cDNA Labeling

1. Combine 14.5 µL total RNA (10 µg) with 1 µL oligo-dTV (5 µg/µL) primer in a 0.2-mL RNAse/DNAse-free PCR tube. 10 µg from each sample where gene expression is being compared is necessary for each reaction (e.g., 10 µg from adult female *S. mansoni* RNA and 10 µg from adult male *S. mansoni* RNA).
2. Heat RNA/oligo-dTV mixture at 70°C for 10 min (it is useful to do all incubation steps in a PCR thermal cycler). Immediately place samples on ice and leave for 5 min. This step effectively removes secondary structures associated with total RNA mixtures.

3. Add 12.6 µL of the master mix containing the amino-allyl dUTP to each denatured RNA/oligo-dTV sample (*see* **Note 15**). Place reactions at 42°C to prewarm samples prior to adding 1 µL Superscript Reverse Transcriptase II (SSRTII). Incubate reactions for 1 h, then add another 1-µL aliquot of SSRTII to each and continue incubating for another hour.
4. Remove cDNA reactions from 42°C and add 10 µL 0.5 *M* EDTA (chelates SSRTII) to each, followed by addition of 10 µL of 1 *N* NaOH (hydrolyzes any remaining RNA). Place samples at 65°C for 15 min and then immediately on ice. Neutralize sample by adding 25 µL of 1 *M* Tris-HCl (7.5).
5. To each neutralized reaction, add 450 µL H_2O and transfer contents into separate Microcon-YM30 concentrators (one for each cDNA reaction). Spin at 13,000*g* in a microcentrifuge for 8 min (*see* **Note 16**). When reservoir volume gets to about 50 µL, add another 400 µL H_2O. Repeat wash with a third 400-µL vol of H_2O (three total washes). These three washes effectively eliminate all unincorporated dNTPs and free Tris (Tris will interfere with Cy dye conjugation reaction).
6. When volume of sample after third wash reaches approx 10 µL, invert the Microcon-YM30 concentrators and place into a new 1.5-mL microcentrifuge tube. Elute washed cDNA samples from filter by spinning at 3000*g* for 4 min.
7. Dry each cDNA sample (will not see dried pellet) in Speed-Vac (time varies depending on temperature).
8. Resuspend each cDNA pellet in 4.5 µL of DNAse-free ddH_2O.
9. Resuspend an aliquot of dried Cy dye (one cDNA sample will be labeled with Cy3 and the other will be labeled with Cy5) in 4.5 µL of 0.1 *M* carbonate/bicarbonate buffer (pH 8.5–9.0) (*see* **Note 17**).
10. Mix cDNA with the appropriate Cy dye and incubate in the dark for 1 h at room temperature (Cy dyes will conjugate to the amino-allyl dUTP that has been incorporated during the cDNA reactions).
11. To stop conjugation reaction, add 4.5 µL of 4 *M* hydroxylamine to each cDNA sample (excess amino groups effectively quench free Cy dye) and incubate in the dark for 15 min.
12. Add 35 µL of 0.1 *M* sodium acetate (pH 5.2) to each conjugation reaction and combine the two samples in which gene expression will be compared. Transfer combined sample to new 1.5-mL microcentrifuge tube.
13. Add 500 µL PB buffer from QIAGEN PCR clean-up kit to each combined Cy3/Cy5-labeled cDNA sample and apply to QIAquick column. Spin samples for 30–60 s, approx 13,000 rpm. Reapply FT to column and spin again. Discard FT (all labeled cDNA should be bound to column), and apply 750 µL PE wash buffer (ensure ethanol has been added to PE buffer as per Qiagen instructions) to column.

AU: Pls convert rpm to g force.

14. Spin columns again for 30–60 s, approx 13,000 rpm. Discard FT. As Cy3 and Cy5 dyes can weakly bind to Qiagen column, it is advisable to perform two additional PE washes (three total). After the third wash, spin for 1 min to completely dry column (*see* **Note 18**).

AU: Pls convert rpm to g force.

15. Place dried column (containing washed, bound, labeled cDNA) in new 1.5-mL collection tube and add 100 µL elution buffer (EB) to membrane. Let sit at room

temperature for 1 min (in dark), then spin for 1 min at 13,000*g* to elute cDNA. Add same volume of EB and repeat elution (collect both fractions in same tube).

16. Bring the 200 µL eluted, labeled cDNA target to 400 µL with DNase-free H_2O and apply to Microcon-YM30 concentrator. Concentrate sample to approx 20–30 µL using a microcentrifuge (*see* **Note 19**). Add 2.2 µL yeast tRNA (4 mg/mL) and 2.2 µL poly d(A) (8 mg/mL) and dry the remaining solution using a Speed-Vac. Resuspend the pellet in 40 µL prewarmed (48°C) filter-sterilized hybridization buffer containing 40% deionized formamide, 5× SSC, 5× Denhart's reagent, 1 m*M* Na-pyrophosphate, 50 m*M* Tris, pH 7.4, 0.1% SDS (*see* **Note 20**).
17. Denature labeled cDNA target for 2 min at 95°C (*see* **Note 20**) and spin for 3 min at 13,000*g* to pellet any insoluble material that may interfere with hybridization. The cDNA target (minus any insoluble pellet) is now ready for hybridization to cDNA microarray.

3.4. cDNA Microarray Preparation, Prehybridization, and Hybridization

cDNA microarrays can be prehybridized for up to 1 h prior to addition of labeled cDNA target. This is an additional cautionary step to prevent nonspecific hybridization events from occurring (may not be needed in all cases).

1. Place slide containing cDNA elements to be used for hybridization in a 50-mL conical tube containing a solution made up of prehybridization buffer (1% BSA/0.1% SDS/5× SSC/50% formamide). Place conical (containing slide) in 42–48°C H_2O bath or hybridization oven, with gentle shaking for 40–50 min.
2. Remove slide from prehybridization conical and briefly wash by immediate immersion and shaking in a Coplin jar containing dH_2O. Repeat wash by transferring slide to another Coplin jar containing an additional aliquot of dH_2O. Finally, submerge slide in a third Coplin jar containing 100% isopropanol.
3. After removal from isopropanol, immediately transfer slides to slide rack and spin dry (do not let air dry) in a benchtop centrifuge (10 min at 1000*g*). Prehybridized slides now can be stored in a clean 50-mL conical tube. When handling slides, touch only sides of glass and always use powder-free gloves. As an alternative to standard glass coverslips, Lifter-slips (coverslips with Teflon-coated raised edges) are commonly used in cDNA microarray hybridizations (Erie Scientific). Lifter-slips facilitate equal distribution of labeled target cDNA across all elements of cDNA microarray.
4. Place the Lifter-slip directly on top of cDNA microarray glass slide with the Teflon-coated raised edges toward the cDNA. Carefully pipet 37 µL of the spun, denatured, labeled cDNA target (leave 3 µL of the target behind so that any insoluble pellet is not disturbed) between Lifter-slip and glass slide and capillary action will draw the liquid to sufficiently cover a fabricated DNA microarray with dimensions of 25 mm × 60 mm (*see* **Note 21**).
5. Place array into hybridization chamber (make sure chamber has a few drops of ddH_2O in each end to maintain humidity), clamp hybridization lid down tightly, and incubate whole apparatus overnight (14–18 h) at 42–50°C.

3.5. cDNA Microarray Washing

After overnight hybridization, slides must be carefully removed from hybridization chambers and washed to remove unbound target DNAs. The washing procedure listed below is a good place to start, however, optimization of washing times, detergent/salt concentrations, and temperature may be necessary. Three Coplin jars are used in this procedure, but any suitable washing tray may be utilized. If using Coplin jars, only two slides should be processed at one time before changing the wash solutions.

1. Remove cDNA microarray from chamber by carefully picking up one end of the glass slide (coverslip still attached) with forceps and place into Coplin jar 1 containing wash solution one (0.5× SSC/0.1% SDS). Carefully shake slide with forceps until coverslip falls off. Remove coverslip from Coplin jar and continue shaking cDNA microarray slide with forceps for 4 min.
2. Remove cDNA microarray from Coplin jar 1, touch end of slide to paper towel to remove excess wash solution (do not allow slide to dry completely), and transfer slide to Coplin jar 2 (containing wash solution two, 0.5X SSC/0.01% SDS). Again, shake slide with forceps for 4 min.
3. Finally, remove cDNA microarray from Coplin jar 2, quickly touch end of slide to paper towel, and transfer slide to Coplin jar 3 (containing wash solution three, 0.06× SSC). Wash slide by vigorous shaking for 4 min as before.
4. Remove cDNA microarray from Coplin jar 3, immediately submerge in 100% isopropanol, and spin dry using a benchtop centrifuge (5 min at 1000*g*). You can now store the cDNA microarray in a dark slide box until ready to acquire fluorescent information via a scanner (processed cDNA arrays can be stored for at least a week in the dark before scanning, without losing data).

3.6. Data Acquisition/Image Analysis/Data Mining/Other Issues

Fluorescent data is acquired through the use of a cDNA microarray scanner (review of some commercially available scanners is available ***[10]***) and appropriate image analysis software. Data mining and filtering (usually the most time-intensive aspect of cDNA microarray experiments) can be performed using limited software packages (Excel®, Microsoft) or quite statistically thorough software packages such as GeneSpring (Silicon Genetics). The specific details about using such equipment and software are beyond the scope of this chapter. Because of the relatively high cost of scanners and software, anyone performing cDNA microarray experiments should consult appropriate references to ascertain which scanner, image analysis software, and data-mining packages are the most suitable for each laboratory's intended experimental setup. It is also recommended that at least three independent hybridization experiments be performed (analytical replication) with each pair of RNA samples to account for intrinsic variables associated with this gene expression platform ***(11)***. Con-

trol experiments are also recommended to assess RNA quality, cDNA labeling, and cDNA microarray hybridization conditions. One control experiment includes performing a "self vs self" hybridization (the two RNA samples are identical, but labeled with different dyes). As both RNA species are the same, this control reveals how tightly a cDNA array estimates that the two samples from the same source produce equivalent signals. Deviation away from normalized gene expression ratio of 1 indicates that the array may be failing in some areas (general or specific). If only some portion of the gene expression ratios is far from 1, then their position on the array can be reassessed (background, signal, etc.) before proceeding with more complicated (and valuable) experimental hybridizations. Another control experiment (very good for bimodal comparisons) involves "dye swapping"—switching the Cy dyes used to label the starting cDNA samples. This control experiment provides additional confidence in gene expression between two samples (the same genes should be differentially expressed even when dyes are switched). Finally, independent confirmation of gene expression, using traditional biological techniques (Northern blotting, RT-PCR, real-time PCR, *in situ* hybridization, protection assays, etc.), should be sought on all differentially expressed cDNAs prior to any detailed advanced investigation.

4. Notes

1. Alternative spotting buffers used by other investigators include SSC (1× through 5× final concentration) or 50% DMSO (with this spotting buffer, generally less evaporation of samples in print plates occur during printing run). Some investigators also add betaine (N,N,N-trimethylglycine: Sigma) to their spotting solution (typically 3× SSC) at a final concentration of 1.5 *M*. This additional spotting compound contributes to high spot homogeneity and reduced background, although slides may need to be baked for longer periods of time (overnight) at 80°C to ensure complete fixation of deposited DNA spots ***(12)***.
2. Typical PCR conditions for amplifying *S. mansoni* cDNA EST inserts from phage suspension or bacteria solution: initial denaturation at 95°C for 5 min; then 35 cycles of 95°C for 30 s, 59°C for 30 s, 72°C for 4 min; final 72°C extension for 5 min; hold at 4°C.

   Phage suspension was prepared as follows: single plaque cored into 500 µL SM buffer and stored frozen (4 µL of this solution was used as template material for PCR).

   Bacterial suspension was prepared as follows: 10 µL from a 1 mL overnight culture was added to 100 µL dH_2O and heated for 5 min at 95°C. This suspension was stored frozen (4 µL of this solution was used as template material for PCR).

   Size of amplified product will undoubtedly vary among ESTs. Generally, 500–2000-bp ESTs are suitable for arraying (more detailed examination of PCR product size on hybridization kinetics and signal intensity has recently been examined ***[13]***).

3. Template DNA generally does not need to be purified prior to PCR amplification. However, in case of particularly difficult PCR amplifications, a template purification step may be necessary. Refer to Sambrook et al. ***(14)*** for general plasmid and phage DNA isolation protocols or access http://www.nhgri.nih.gov/DIR/Microarray/main.html or http://www.microarrays.org for typical microarray template purification procedures.
4. Centrifugation may replace the use of a vacuum manifold if necessary. In this case, centrifuge 96-well filter plates containing PCR products at 4500*g* for 10–15 min in substitution of vacuum application.
5. It is critical to shake moderately (not vigorously) to elute DNA samples from the membrane and also to prevent cross-contamination from well to well. Evaluate the rotation speed before elution with a water/dye combination. If you wish to obtain PCR products at a higher concentration, simply elute with less volume. DNA in a range of 0.15–0.25 mg/mL is a good starting concentration for robotic printing (concentration estimates can be made from gel electrophoresis). Alternatively, a precipitation step may be performed using isopropanol, according to protocols at http://www.microarrays.org. Excessively concentrated DNA may clog pins or produce "comet tails" (caused by localized reattachment of excess spot material to the slide surface during postprinting processing), whereas DNA below this concentration leads to weak signal and a decrease in the dynamic range for detecting differentially expressed genes. Refer to Yue et al. (***15***), http://www.nhgri.nih.gov/DIR/Microarray/main.html, for methods of determining precise starting DNA concentration in source plate (typically a fluorescent assay that can be performed in 96-well plates). Additionally, SYBR GREEN II RNA gel stain (Molecular Probes) can be used as quick test to determine how efficiently the DNA has been deposited onto each microarray prior to hybridization (protocol available at http://www.probes.com/handbook/boxes/0681.html).
6. A review of the specifications and features of some commercially available robotic printers or arrayers is provided by D. Bowtell ***(10)***. Additionally, an alternative to printing PCR products onto γ-amino propyl silane-coated slides is to print onto poly-*L*-lysine-coated slides. Both coating agents essentially perform the same function—provide a hydrophobic and positive charge critical for spotting and positioning of DNA. Methods and reagents to coat Gold Seal brand microscope slides (this particular glass has minimal intrinsic autofluorescence—Erie Scientific) can be found at http://www.nhgri.nih.gov/DIR/Microarray/main.html. Although coating microscope slides in house with poly-*L*-lysine is more cost-effective than purchasing either poly-*L*-lysine or γ-amino propyl silane-coated slides, batch-to-batch variation in slide coating often can occur if care is not taken in quality control. There are many varieties of printing tools and pins (split pins are commonly used), and the pros and cons of each make are beyond the scope of this chapter. Consult an experienced company (e.g., Biorobotics or TeleChem) for details of each tool and pin type.
7. In cases of high background, an extra slide-blocking step can be performed with succinic anhydride (Corning; www.corning.com, provides instructions with their

γ-amino propyl silane-coated slides, or alternatively, instructions can be found at http://www.nhgri.nih.gov/DIR/Microarray/main.html). Succinic anhydride converts the residual amines on the surface of the precoated slide into carboxylic moieties (reduces the positive charge of non-DNA-containing areas and, therefore, limits the areas in which cDNA can bind nonspecifically). New techniques ***(12)*** have improved this blocking procedure by effectively limiting the amount of DNA solubilization that sometimes occurs during succinic anhydride treatment.

8. The correct volume of TRIZOL reagent added to initial parasite sample should be empirically determined for optimal yields of RNA.
9. In some cases (multicellular, multitissue parasites such as adult nematodes and helminths, etc.), a more vigorous shaking step is suggested (3 min instead of just 1 min).
10. If volume of material is too large to spin in a microcentrifuge, use a Sorvall RC-5B (or equivalent) centrifuge and spin at 6,000–10,000*g* increasing the time to 30 min (Sorvall HB-6 rotor). This centrifugation step will require RNAse-free 15- or 30-mL Corex tubes (treated with 0.1 *M* HCl in DEPC-H_2O for at least 8 h and subsequently autoclaved).
11. Occasionally, during this step some precipitation of nucleic acid (when starting with approx 1 g of material) is observed. This does not appear to interfere with the subsequent binding of RNA to the Qiagen column as long as the sample is thoroughly vortexed prior to addition to column.
12. The three successive elution volumes are based on the size (midi or maxi) of the Qiagen column used. Suggested elution volumes for each column are indicated in the supplied manufacturer's handbook.
13. As RNA isolated in this manner is free of contaminating carbohydrate molecules (carbohydrates bound to RNA often makes RNA difficult to resuspend), complete Speed-Vac drying of the sample generally does not inhibit the subsequent solubility of each sample.
14. RNA is typically diluted 1/100 in 0.01 *M* Tris-HCl (7.5) for estimations of purity (OD_{260}:OD_{280} ratio). An OD_{260}:OD_{280} ratio of 2.0–2.3 (in 0.01 *M* Tris-HCl) generally indicates highly pure RNA free of contamination. For accurate RNA quantitation, measure samples in RNAse-free H_2O.
15. The cDNA master mix in this protocol used a 2:3 ratio of aa-dUTP to dTTP. This ratio works quite well for schistosome RNA. However, empirical determination of the correct ratio for each parasite species is essential for optimal labeling efficiency (this ratio should be a good starting point for optimization experiments).
16. Depending on the microcentrifuge being used as well as fine differences in microcon membrane construction, the time spent washing the cDNA samples may vary. Try to avoid drying the microcon membrane completely, as some difficulty may be encountered when eluting the cDNA (although according to the manufacturer's instructions, 10 μL of H_2O can be applied to dried membranes, allowing for recovery of cDNA sample material).
17. It is important not to let the Cy dyes sit in an aqueous buffer prior to conjugation, only rehydrate the dyes directly prior to addition of suspended cDNA sample. Small residual amounts of DMSO do not affect conjugation reaction.

18. Strong color will be observed in the FT following the first wash. This material represents unconjugated dyes. Some color may also be noticed on the Qiagen membrane after spin drying of the samples (after the third wash). This material likely represents trapped Cy3/Cy5-labeled SSRT II (free amino groups on the enzyme capable of being modified by the dyes during the conjugation reaction) and/or labeled cDNA targets. With 10 μg input RNA, one does not consistently observe color on the membrane prior to elution of cDNA or in the eluent.
19. It can be useful to include either human or mouse Cot-1 DNA (1 μg Cot-1 DNA/10 μg input RNA) during this concentration step as an additional blocking agent in schistosome cDNA microarray hybridizations. Cot-1 DNA is 50–310 bp in size and enriched for repetitive DNA sequences such as Alu and Kpn (human Cot-1) or B1, B2, and L1 (mouse Cot-1) family members. Including one of these blocking agents prevents undesired hybridization between conserved repetitive DNA sequences in the labeled cDNA and probe DNA by competitive hybridization. Other blocking agents can be added during hybridization, such as yeast tRNA and poly d(A). Yeast tRNA reduces undesired nonspecific DNA hybridization, while poly d(A) that is 40–60 dATP bases in length promotes specific hybridization between the labeled cDNA and probe DNA. Poly d(A) reduces hybridization of the polyA sequences in the probe DNA to the polyT tract in the labeled cDNA.
20. This hybridization buffer is one that works well for schistosome cDNA arrays, although each user should empirically test different components (blocking agents) and formulations (formamide vs aqueous based hybridization buffers) to ensure optimal hybridization kinetics. An aqueous hybridization buffer may also be used consisting of 3.5× SSC/0.275% SDS. In this case, the hybridization is carried out at 65°C overnight instead of 42–50°C. However, dehydration of labeled cDNA sample material between coverslip and cDNA microarray may occasionally occur when using an aqueous hybridization buffer. Using either hybridization buffer, do not place labeled cDNA target on ice after heating at 95°C, as SDS will precipitate.
21. A glass coverslip/Lifter-slip can be cleaned of dust and debris by using compressed air. Ensure that the compressed-air canister does not also spray oil droplets or other potential contaminants onto the glass coverslip. Alternatively, the coverslip/Lifter-slip may be cleaned using EtOH. Care should be taken not to introduce air bubbles between glass coverslips and cDNA microarrays. If air bubbles are observed, then carefully push bubbles to the side of the coverslip with clean forceps prior to hybridization. Small air bubbles will usually dissipate during the hybridization.

Acknowledgments

We thank Drs. Rhian Hayward and Michael Wilson for critically reviewing this chapter. We also thank Drs. David Latto, Tom Freeman, David Johnston, and David Dunne for many helpful discussions concerning cDNA microarray applications for schistosome genomic investigations.

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